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A METHOD OF IMPROVING EFFICACY OF BIOLOGICAL RESPONSE-MODIFYING PROTEINS AND THE EXEMPLARY MUTEINS

Technical Field

The present invention relates to a protein variant which substitutes valine for phenylalanine residue in a binding domain having a biological response-modifying function by binding to a receptor, ligand or substrate. More particularly, the present invention relates to a protein variant which substitutes valine for phenylalanine residue in an α-helix domain participating in the binding of a human cytokine protein to a corresponding receptor.

Background Art

Many human diseases are caused by the loss of protein function due to defects or an insufficient amount of a protein. To treat such diseases, related proteins have been directly administered to patients. However, many physiologically active proteins used as medicines are easily degraded in serum before they arrive at target tissues and act therein. For this reason, most physiologically active proteins having therapeutic value are excessively or frequently administered to patients to maintain an appropriate concentration capable of offering satisfactory therapeutic effects.

An approach to solve the above problems is to conjugate with polyethylene glycol (PEGylation) or microencapsulate physiologically active proteins. However, these methods are cumbersome because target proteins are primarily produced in microorganisms and purified, and are then PEGlyated or microencapsulated. In addition, cross-linking may occur at undesired positions, which may negatively affect the homogeneity of final products.

Another approach involves glycosylation. Cell surface proteins and secretory proteins produced by eukaryotic cells are modified by a glycosylation process. Glycosylation is known to influence in vivo stability and function of proteins, as well as their physiological properties. However, since glycosylated proteins can be produced only by eukaryotic cells capable of

performing glycosylation, their production process is complicated, and it is difficult to attain homogeneous final products which are glycosylated at all desired positions.

In addition, the conventional techniques all improve the problems associated with administration frequency, but do not increase the physiological efficacy of proteins, leading to excessive dosage. For example, NESP developed by the Amgen Company (see U.S. Pat. No. 6,586,398) improves the frequent administration by extending the half-lives of proteins in the blood, but does not increase the efficacy of proteins, leading to excessive dosage that may induce the production of blocking antibodies.

An approach used to improve the efficacy of physiologically active proteins is to mutagenize some amino acid residues of a wild-type protein to improve biological activity of the protein. Related protein variants are disclosed in the following patent publications: (1) U.S. Pat. No. 5,457,089: human erythropoietin (EPO) variants where the carboxyl terminal region was altered to increase binding affinity of EPO to its receptor; (2) International Pat. Publication No. 02/077034: human granulocyte colony stimulating factor (G-CSF) variants where a T-cell epitope was altered to reduce immunogenicity of human G-CSF in humans; (3) International Pat. Publication No. 99/57147: human thrombopoietin (TPO) variants prepared by substuting glutaminic acid at the 115 position with lysine, arginine or tyrosine in a TOP protein having an amino acid sequence corresponding to 7th to 151st amino acid residues of human mature TPO; and (4) U.S. Pat. Nos. 6,136,563 and 6,022,711 that disclose human growth hormone variants having alanine substitutions at the 18, 22, 25, 26, 29, 65, 168 and 174 positions.

However, the aforementioned protein variants are altered forms made for improving only therapeutic efficacy regardless of changes in in vivo antigenicity. Thus, the scale, degree and position of these alterations have high potential to induce immune responses in humans. Antigenicity in humans may cause serious adverse effects (Casadevall et al. N. Eng. J. Med. 2002, vol.346, p.469).

Disclosure of the Invention

It is therefore an object of the present invention to provide biological response-modifying

protein variants having improved pharmacological action, which are capable of maximizing biological response modifying effects upon administration and preventing the formation of blocking antibodies through an improvement in efficacy of conventional biological response-modifying proteins, and methods of preparing such variants.

In one aspect, the present invention provides a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate.

In another aspect, the present invention provides a DNA encoding a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate.

In a further aspect, the present invention provides a recombinant expression vector to which a DNA encoding a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate is operably linked.

In yet another aspect, the present invention provides a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate is operably linked.

In still another aspect, the present invention provides a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate is operably linked, and isolating the protein variant from a resulting culture.

In still another aspect, the present invention provides a pharmaceutical composition comprising a protein variant which substitutes valine for phenylalanine residue in a binding domain of a protein having a biological response-modifying function by binding to a receptor, ligand or substrate, and a pharmaceutically acceptable carrier.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1A is a multiple alignment of amino acid sequences of domains participating in the binding of 4-helix bundle cytokines to corresponding receptors;

FIG. 1B is a multiple alignment of amino acid sequences of domains participating in the binding of interferons to corresponding receptors;

FIG. 2A shows the results of Western blotting of TPO variants according to the present invention, (from the leftmost lane: marker; wild-type TPO; TPO-[F46V]; TPO-[F128V]; TPO-[F131V]; and TPO-[F141V]);

FIG. 2B shows the results of Western blotting of EPO variants according to the present invention, (from the leftmost lane: marker; wild-type EPO; EPO-[F48V]; EPO-[F138V]; EPO-[F142V]; and EPO-[F148V]);

FIG. 2C shows the results of Western blotting of G-CSF variants according to the present invention, (from the leftmost lane: marker; wild-type G-CSF; G-CSF-[F13V]; G-CSF-[F83V]; G-CSF-[F113V]; G-CSF-[F140V]; G-CSF-[F144V]; and G-CSF-[F160V]);

FIG. 3A is a graph showing the relative expression levels of TPO variants according to the present invention, compared to a wild-type TPO;

FIG. 3B is a graph showing the relative expression levels of EPO variants according to the present invention, compared to a wild-type EPO;

FIG. 3C is a graph showing the relative expression levels of G-CSF variants according to the present invention, compared to a wild-type G-CSF;

FIG. 4A shows the results of an ELISA assay for binding affinity of TPO variants according to the present invention to TPO receptors;

FIG. 4B shows the results of an ELISA assay for binding affinity of EPO variants according to the present invention to EPO receptors;

FIG. 4C shows the results of an ELISA assay for binding affinity of G-CSF variants according to the present invention to G-CSF receptors;

FIG. 4D shows the results of an ELISA assay for binding affinity of GH variants according to the present invention to GH receptors;

FIG. 5A shows the results of an SPR assay for binding affinity of TPO variants according to the present invention to TPO receptors;

FIG. 5B shows the results of an SPR assay for binding affinity of EPO variants according to the present invention to EPO receptors;

FIG. 6A shows the results of a FACS analysis for binding affinity of a TPO variant according to the present invention to TPO receptors;

FIG. 6B shows the results of a FACS analysis for binding affinity of an EPO variant according to the present invention to EPO receptors;

FIG. 7A is a graph showing the proliferation rates of TF-1/*c-Mpl* cells according to the concentration of TPO variants according to the present invention;

FIG. 7B is a graph showing the proliferation rates of TF-1 cells according to the concentration of EPO variants according to the present invention;

FIG. 7C is a graph showing the proliferation rates of HL60 cells according to the concentration of G-CSF variants according to the present invention;

FIG. 7D is a graph showing the proliferation rates of Nb2 cells according to the concentration of GH variants according to the present invention;

FIG. 8A is a graph showing the results of a pharmacokinetic assay of a TPO variant according to the present invention, in which the TPO variant was intravenously injected into rabbits, and serum levels of the TPO variant were measured;

FIG. 8B is a graph showing the results of a pharmacokinetic assay of an EPO variant according to the present invention, in which the EPO variant was intravenously injected into rabbits, and serum levels of the EPO variant were measured;

FIG. 8C is a graph showing the results of a pharmacokinetic assay of an EPO variant according to the present invention, in which the EPO variant was intraperitoneally injected into mice,

and serum levels of the EPO variant were measured;

FIGS. 9A, 9B and 9C are graphs showing the proliferation rates of erythrocytes, proliferation rates of reticulocytes, and changes in hematocrit, respectively, as results of tests to evaluate in vivo activity of EPO variants according to the present invention, in mice intraperitoneally injected with the EPO variants; and

FIGS. 10A, 10B and 10C are graphs showing proliferation rates of platelets, leukocytes and neutrophils, respectively, as results of tests to evaluate the in vivo activity of TPO variants according to the present invention, in rats intraperitoneally injected with the TPO variants.

Best Mode for Carrying Out the Invention

Single capital letters standing for amino acids, as used herein, represent the following amino acids according to the standard abbreviations defined by the International Union of Biochemistry:

A: Alanine; B: Asparagine or Aspartic acid;

C: Cysteine; D: Aspartic acid; E: Glutamic acid;

F: Phenylalanine; G: Glycine; H: Histidine;

I: Isoleucine; K: Lysine; L: Leucine;

M: Methionine; N: Asparagine; P: Proline;

Q: Glutamine; R: Arginine; S: Serine;

T: Threonine; V: Valine; W: Tryptophan;

Y: Tyrosine; and Z: Glutamine or Glutamic acid.

The designation "(one capital for an amino acid)(amino acid position)(one capital for another amino acid)", as used herein, means that the former amino acid is substituted by the latter amino acid at the designated amino acid position of a certain protein. For example, F48V indicates that the phenylalanine residue at the 48th position of a certain protein is substituted by valine. The amino acid position is numbered from the N terminus of a mature wild-type protein.

The term "protein variant", as used herein, refers to a protein that has an amino acid

sequence different from a wild-type form by a substitution of valine for phenylalanine residue in a protein having physiological function by binding to a receptor, ligand or substrate, in particular, in a domain participating in the binding to a receptor, ligand or substrate. In the present invention, a protein variant is designated for convenience as "protein name-[(one capital for an amino acid)(amino acid position)(one capital for another amino acid)]". For example, TPO-[F131V] indicates a TPO variant in which the phenylalanine residue at position 131 of wild-type TPO is substituted by valine.

The term "biological response-modifying proteins", as used herein, refers to proteins involved in maintaining homeostasis in the body by inducing the initiation or stop of various biological responses occurring in the multicellular body and regulating the responses to be organically connected to each other. These proteins typically act by binding to receptors, ligands or substrates.

Proteins capable of being altered according to the present invention include all proteins that have innate function to modulate biological responses by binding receptors, ligands or substrates. Non-limiting examples of the proteins include cytokines, cytokine receptors, adhesion molecules, tumor necrosis factor (TNF) receptors, enzymes, receptor tyrosine kinases, chemokine receptors, other cell surface proteins, and soluble ligands. Non-limiting examples of the cytokines include CNTF (cytoneurotrophic factor), GH (growth hormone), IL-1, IL-1Ra (interleukin-1 receptor antagonist), placental lactogen (PL), cardioliphin, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-17, TNF, TGF (transforming growth factor), IFN (interferon), GM-CSF (granulocyte-monocyte colony stimulating factor), G-CSF (granulocyte colony stimulating factor), EPO (erythropoietin), TPO (thrombopoietin), M-CSF (monocyte colony stimulating factor), LIF (leukemia inhibitory factor), OSM (oncostatin-M), SCF (stem cell factor), HGF (hepatocyte growth factor), FGF (fibroblast growth factor), IGF (insulin-like growth factor), and LPT (Leptin). Non-limiting examples of the cytokine receptors include growth hormone receptor (GHR), IL-13R, IL-1R, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-15R, TNFR, TGFR, IFNR (e.g., IFN-γR α-chain, IFN-γR β-chain), interferon-αR, -βR and -γR, GM-CSFR, G-CSFR, EPOR, cMpl, gp130, and Fas (Apo 1). Examples of the chemokine receptors include CCR1 and CXCR1-4. Examples of the receptor tyrosine kinases include TrkA, TrkB, TrkC, Hrk, REK7, Rse/Tyro-3, hepatocyte growth factor R, platelet-derived growth factor R, and Flt-1. Examples of other cell surface proteins include

CD2, CD4, CD5, CD6, CD22, CD27, CD28, CD30, CD31, CD40, CD44, CD100, CD137, CD150, LAG-3, B7, B61, β-neurexin, CTLA-4, ICOS, ICAM-1, complement R-2(CD21), IgER, lysosomal membrane gp-1, α2-microglobulin receptor-related protein, and natriuretic peptide receptor.

To improve the efficacy of modulating biological responses for the aforementioned numerous proteins having biological response-modulating function, the present invention intends to provide protein variants capable of binding to receptors, ligands or substrates having a higher hydrophobic force than that of wild types. For this purpose, the present invention is characterized by substituting valine for phenylalanine residue in a binding domain of each of the proteins.

Phenylalanine is a relatively non-polar amino acid that has an aromatic side chain and a known hydrophobicity index of 3.0. Valine is a non-polar hydrophobic amino acid that has an aliphatic side chain and a known hydrophobicity index of 4.0. In addition, since valine is smaller than phenylalanine, a protein substituting valine for phenylalanine residue becomes more deeply depressed in a pocket binding to a corresponding receptor, ligand or substrate. Thus, a protein substituting valine for phenylalanine residue in a binding domain has increased hydrophobic force and a more deeply depressed space so that it has increased binding affinity to a receptor, ligand or substrate, leading to a desired increase in biological response-modulating efficiency.

In addition, the valine substitution for phenylalanine residue, as a conservative substitution, has a minimal influence on the secondary or tertiary structure of a protein, and thus rarely affects the function of the protein (Argos, EMBO J. 1989, vol.8, pp779-85). Further, because phenylalanine is mainly present in a highly hydrophobic region, it is rarely exposed to the exterior. When such phenylalanine residue is substituted by valine, a protein becomes more deeply depressed from the surface due to the higher hydrophobicity of valine. Thus, this substitution has a lower potential to induce antibody production. A certain protein should primarily bind a corresponding receptor, ligand or substrate to modulate a specific biological response. In the case that the stronger this binding is, the efficacy of modulating a biological response is improved, related proteins all may be altered according to the present invention, and the present invention includes all of the resulting protein variants.

The fact that such a substitution of valine for phenylalanine residue leads to increased

binding affinity is supported by the finding of a mutation of FcγRIIIa(CD16) expressed on NK cells in human autoimmune diseases. The human receptor protein has a genetic polymorphism. That is, individuals are divided into two groups: at position 176 in a region participating in recognizing Fc of an antibody ligand, one group has phenylalanine, and the other group has valine. Individuals having phenylalanine at position 176 of the receptor have weakened binding affinity to the Fc region of the antibody ligand and are highly susceptible to systemic lupus erythematosus (SLE) (Jianming Wu et al. J. Clin. Invest. 1997, vol.100, pp.1059-70).

On the other hand, as noted above, the present invention is characterized by substituting valine for phenylalanine residue in a binding domain of a biological response-modulating protein. The term "binding domain", as used herein, refers to a portion (that is, domain) of a protein performing its biological function by binding to a receptor, ligand or substrate, and has relatively high hydrophobicity and low antigenicity compared to other regions of the protein. Binding domains of proteins are well known in the art. For example, some 4-α helix bundle cytokines and interferons, which are used in an embodiment of the present invention, are known to have a D-α helix structure and an A-α helix structure, respectively, that serve as binding domains for corresponding receptors.

However, a binding domain altered according to the present invention is not limited to binding domains known in the art. This is because the binding of a biological response-modulating protein to a receptor, ligand or substrate is influenced by, in addition to amino acid residues involved in direct binding, other several amino acid residues. A "binding domain" of a biological response-modulating protein, altered according to the present invention, further includes about 50 amino acid residues, preferably about 25 amino acid residues, and more preferably about 10 amino acid residues, from both ends of a binding domain known in the art.

One aspect of the present invention involves cytokines that typically contain several α helix structures. Among them, the first and last helices from the N-termius are known as binding domains participating in binding of cytokines to corresponding cytokine receptors (see FIG. 1). α helices responsible for binding of cytokines to corresponding receptors differ according to the type of cytokines, and are well known in the art. For example, in IL-2, the second and fifth helices bind to the p55α receptor among IL-2 receptors, the first helix binds to the p75γ receptor among IL-2

receptors, and the sixth helix binds to gamma receptor (Fernando Bazan, Science J. 1992, vol.257, pp.410-2). As described above, cytokines each have particular helices participating in binding, but the helices have highly conserved amino acid sequences. The present invention provides a cytokine variant that is capable of binding to a cytokine receptor with higher affinity than a wild-type cytokine by substituting valine for phenylalanine residue in an alpha helix corresponding to a binding domain of a cytokine.

One aspect related to the cytokines involves the 4-helix bundle family of cytokines. Such cytokines include CNTF, EPO, Flt3L, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p35, LPT, LIF, M-CSF, OSM, PL, SCF, TPO G-CSF, GHR and IFN. These cytokines all have four alpha helices, which are designated as A-alpha helix, B-alpha helix, C-alpha helix and D-alpha helix, respectively. The D- and A-alpha helices mainly participate in binding to receptors (Fernando Bazan, Immunology today, 1990, vol.11 pp.350-4, The Cytokine Facts Book, 1994, pp.104-247).

Among the aforementioned 4-helix bundle cytokines, CNTF, EPO, Flt3L, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p35, LPT, LIF, M-CSF, OSM, PL, SCF, TPO, G-CSF and GHR have binding domains which each include a D-alpha helix and a region linking a C-alpha helix and the D-alpha helix. More particularly, the binding domains include amino acid residues between positions 110 and 180 among amino acid residues of the 4-helix bundle cytokines. Therefore, in an aspect, the present invention provides a 4-helix bundle cytokine variant that is capable of binding to a corresponding receptor with higher affinity than a wild type by substituting valine for phenylalanine among amino acid residues between positions 110 and 180 of a 4-helix bundle cytokine.

Of the aforementioned 4-helix bundle cytokines, interferons (e.g., IFN-α2A, IFN-α2B, IFN-β, IFN-γ, IFN-ω, IFN-τ) have a binding domain that contains an "A-alpha helix". More particularly, the binding domain of interferons includes amino acid residues between positions 1 and 50. Therefore, in another aspect, the present invention provides an interferon variant that is capable of binding to an interferon receptor having higher affinity than a wild type by substituting valine for phenylalanine among amino acid residues between positions 1 and 50 of an interferon.

On the other hand, the binding domain altered according to the present invention may include two or more phenylalanine residues. The two or more phenylalanine residues may all be substituted

by valine. However, because this case leads to a great reduction in protein expression levels, preferably only one phenylalanine residue is substituted by valine. In this regard, the present inventors found that, when phenylalanine residue present in a highly hydrophobic region is substituted by valine, the biological response-modulating protein has much improved efficacy. Therefore, in the present invention, the phenylalanine residue to be substituted by valine is preferably selected in a highly hydrophobic region present in the binding domain specified according to the present invention. Hydrophobicity for a specific region of an amino acid sequence comprising a protein may be determined by a method known in the art (Kyte, J. et al. J. Mol. Biol. 1982, vol.157, pp.105-132, Hopp, T.P. et al. Proc. Nat. Acad. Sci. USA, 1981, vol. 78(6), pp.3824-3828).

The variant of a biological response-modulating protein according to the present invention may be prepared by chemical synthetic methods generally known in the art (Creighton, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY 1983). Representative methods, but are not limited to, include liquid or solid phase synthesis, fragment condensation, and F-MOC or T-BOC chemical synthesis (Chemical Approaches to the Synthesis of Peptides and Proteins, Williams et al., Eds., CRC Press, Boca Raton Florida, 1997; A Practical Approach, Atherton & Sheppard, Eds., IRL Press, Oxford, England, 1989).

Alternatively, the protein variant according to the present invention may be prepared by recombinant DNA techniques. These techniques include a process of preparing a DNA sequence encoding the protein variant according to the present invention. Such a DNA sequence may be prepared by altering a DNA sequence encoding a wild-type protein. In brief, after a DNA sequence encoding a wild-type protein is synthesized, a codon for phenylalanine is changed to another codon for valine by site-directed mutagenesis, thus generating a desired DNA sequence.

Also, the preparation of a DNA sequence encoding the protein variant according to the present invention may be achieved by a chemical method. For example, a DNA sequence encoding the protein variant may be synthesized by a chemical method using an oligonucleotide synthesizer. An oligonucleotide is made based on an amino acid sequence of a desired protein variant, and preferably by selecting a appropriate codon used by a host cell producing a protein variant. The degeneracy in the genetic code, which means that one amino acid is specified by more than one

codon, is well known in the art. Thus, there is a plurality of DNA sequences with degeneracy encoding a specific protein variant, and they all fall into the scope of the present invention.

A DNA sequence encoding the protein variant according to the present invention may or may not include a DNA sequence encoding a signal sequence. The signal sequence, if present, should be recognized by a host cell selected for the expression of the protein variant. The signal sequence may have a prokaryotic or eukaryotic origin or a combinational origin, and may be a signal sequence of a native protein. The employment of a signal sequence may be determined according to the effect of expression of a protein variant as a secretory form in a recombinant cell producing the protein variant. If a selected cell is a prokaryotic cell, a DNA sequence typically does not encode a signal sequence but instead contains preferably an N-terminal methionine for direct expression of a desired protein, and most preferably, a signal sequence derived from a wild type protein is used.

Such a DNA sequence as prepared above is operably linked to another DNA sequence encoding the protein variant of the present invention, and is inserted into a vector including one or more expression control sequences regulating the expression of the resulting DNA sequence. Then, a host is transformed or transfected with the resulting recombinant expression vector. The resulting transformant or transfectant is cultured in a suitable medium under suitable conditions for the expression of the DNA sequence. A substantially pure variant of a biological response-modulating protein coded by the DNA sequence is recovered from the resulting culture.

The term "vector", as used herein, means a DNA molecule serving as a vehicle capable of stably carrying exogeneous genes into host cells. For useful application, a vector should be replicable, have a system for introducing itself into a host cell, and possess selectable markers. In addition, the term "recombinant expression vector", as used herein, refers to a circular DNA molecule carrying exogeneous genes operably linked thereto to be expressed in a host cell. When introduced into a host cell, the recombinant expression plasmid has the ability to replicate regardless of host chromosomal DNA at a high copy number and to produce heterogeneous DNA. As generally known in the art, in order to increase the expression level of a transfected gene in a host cell, the gene should be operably linked to transcription and translation regulatory sequences functional in a host cell selected as an expression system. Preferably, the expression regulation sequences and the

exogeneous genes may be carried in a single expression vector containing bacteria-selectable markers and a replication origin. In the case that eukaryotic cells are used as an expression system, the expression vector should further comprise expression markers useful in the eukaryotic host cells.

The term "expression control sequences", as used herein in connection with a recombinant expression vector, refers to nucleotide sequences necessary or advantageous for expression of the protein variant according to the present invention. Each control sequence may be native or foreign to the nucleotide sequence encoding the protein variant. Non-limiting examples of the expression control sequences include leader sequences, polyadenylation sequences, propeptide sequences, promoters, enhancers or upstream activating sequences, signal peptide sequences, and transcription terminators. The expression control sequence contains at least one promoter sequence.

The term "operably linked" refers to a state in which a nucleotide sequence is arranged with another nucleotide sequence in a functional relationship. The nucleotide sequences may be a gene and control sequences, which are linked in such a manner that gene expression is induced when a suitable molecule (for example, transcription-activating protein) binds to the control sequence(s). For example, when a pre-sequence or secretory leader facilitates secretion of a mature protein, it is referred to as "operably linked to the protein". A promoter is operably linked with a coding sequence when it regulates transcription of the coding sequence. A ribosome-binding site is operably linked to a coding sequence when it is present at a position allowing translation of the coding sequence. Typically, the term "operably linked" means that linked nucleotide sequences are in contact with each other. In the case of a secretory leader sequence, the term means that it contacts a coding sequence and is present within a leading frame of the coding sequence. However, an enhancer need not necessarily contact a coding sequence. Linkage of the nucleotide sequences may be achieved by ligation at convenient restriction enzyme recognition sites. In the absence of restriction enzyme recognition sites, oligonucleotide adaptors or linkers may be used, which are synthesized by the conventional methods.

In order to express a DNA sequence encoding the protein variant according to the present invention, a wide variety of combinations of host cells and vectors as an expression system may be used. Expression vectors useful for transforming eukaryotic host cells contain expression regulation sequences from, for example, SV40, bovine papillomavirus, adenovirus, adeno-associated viruses,

cytomegalovirus and retroviruses. Expression vectors useful in bacterial host cells include bacterial plasmids from *E. coli*, which are exemplified by pET, pRSET, pBluescript, pGEX2T, pUC, pBR322, pMB9 and derivatives thereof, plasmids having a broad range of host cells, such as RP4, phage DNAs, exemplified by a wide variety of λ phage derivatives including λ gt10, λ gt11 and NM989, and other DNA phages, exemplified by filamentous single-stranded DNA phages such as M13. Expression vectors useful in yeast cells include 2μ plasmid and derivatives thereof. Expression vectors useful in insect cells include pVL 941.

To express a DNA sequence encoding the protein variant according to the present invention, any of a wide variety of expression control sequences may be used by these vectors. Such useful expression control sequences include those associated with structural genes of the aforementioned expression vectors. Examples of useful expression control sequences include the early and later promoters of SV40 or adenoviruses, the lac system, the trp system, the TAC or TRC system, T3 and T7 promoters, the major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of phosphatases, for example, Pho5, the promoters of the yeast alpha-mating system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. In particular, T7 RNA polymerase promoter Φ 10 is useful for expressing a polypeptide in E. *coli*.

Host cells transformed or transfected with the aforementioned recombinant expression vector comprise another aspect of the present invention. A wide range of mononuclear host cells may be used for expressing a DNA sequence encoding the protein variant of the present invention. Examples of the host cells include prokaryotic and eukaryotic cells such as *E. coli*, *Pseudomonas* sp., *Bacillus* sp., *Streptomyces* sp., fungi or yeasts, insect cells such as *Spodoptera frugiperda* (Sf9), animal cells such as Chinese hamster ovary cells (CHO) or mouse cells, African green monkey cells such as COS 1, COS 7, BSC 1, BSC 40 or BMT 10, and tissue-cultured human and plant cells. Preferred hosts include bacteria such as *E. coli* and *Bacillus subtilis*, and tissue-cultured mammalian cells.

The transformation and transfection may be performed by the methods described in basic experimental guidebooks (Davis et al., Basic Methods in Molecular Biology, 1986; Sambrook, J., et al.,

Basic Methods in Molecular Biology, 1989). The preferred methods for introducing a DNA sequence encoding the protein variant according to the present invention into a host cell include, for example, calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, and infection.

Also, it will be understood that all vectors and expression control sequences do not function equally in expressing the DNA sequence of the present invention. Likewise, all hosts do not function equally for an identical expression system. However, those skilled in the art are able to make a suitable selection from various vectors, expression control sequences and hosts, within the scope of the present invention, without a heavy experimental burden. For example, a vector may be selected taking a host cell into consideration because the vector should be replicated in the host cell. The copy number of a vector, ability to control the copy number, and expression of other proteins encoded by the vector, for example, an antibiotic marker, should be deliberated. Also, an expression control sequence may be selected taking several factors into consideration. For example, relative strength, control capacity and compatibility with the DNA sequence of the present invention of the sequence, particularly with respect to possible secondary structures, should be deliberated. Further, the selection of a host cell may be made under consideration of compatibility with a selected vector, toxicity of a product encoded by a nucleotide sequence, secretory nature of the product, ability to correctly fold a polypeptide, fermentation or cultivation requirements, ability to ensure easy purification of a product encoded by a nucleotide sequence, or the like.

In the method of preparing the protein variant according to the present invention, the host cells are cultivated in a nutrient medium suitable for production of a polypeptide using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, small-scale or large-scale fermentation in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium containing carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are commercially available from commercial suppliers and may be prepared according to published compositions (for example, the catalog of American Type Culture

Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The biological response-modulating protein variant according to the present invention may be recovered by methods known in the art. For example, the protein variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation. Further, the protein variant may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobicity, and size exclusion), electrophoresis, differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction.

The present invention provides a pharmaceutical composition comprising a variant of a biological response-modulating protein and a pharmaceutically acceptable carrier. In the pharmaceutical composition according to the present invention, the biological response-modulating protein variant is preferably contained in a therapeutically effective amount.

The carrier used in the pharmaceutical composition of the present invention includes the commonly used carriers, adjuvants and vehicles, in the pharmaceutical field, which are as a whole called "pharmaceutically acceptable carriers". Non-limiting pharmaceutically acceptable carriers useful in the pharmaceutical composition of the present invention include ion exchange, alumina, aluminum stearate, lecithin, serum proteins (e.g., human serum albumin), buffering agents (e.g., sodium phosphate, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of vegetable saturated fatty acids), water, salts or electrolytes (e.g., protamine sulfate, disodium hydrophosphate, potassium hydrophoshate, sodium chloride, and zinc salts), colloidal silica, magnesium trisilicate, polyvinylpyrrolidone, cellulose-based substrates, polyethylene glycol, sodium carboxymethylcellulose, polyarylate, waxes, polyethylene-polyoxypropylene-block copolymers, polyethylene glycol, and wool fat.

The pharmaceutical composition of the present invention may be administered via any of the common routes, if it is able to reach a desired tissue. Therefore, the pharmaceutical composition of the present invention may be administered topically, orally, parenterally, intraocularly, transdermally, intrarectally and intraluminally, and may be formulated into solutions, suspensions, tablets, pills,

capsules and sustained release preparations. The term "parenteral", as used herein, includes subcutaneous, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intra-synovial, intrasternal, intracardial, intrathecal, intralesional and intracranial injection or infusion techniques.

In an aspect, the pharmaceutical composition of the present invention may be formulated as aqueous solutions for parenteral administration. Preferably, a suitable buffer solution, such as Hank's solution, Ringer's solution or physiologically buffered saline, may be employed. Aqueous injection suspensions may be supplemented with substances capable of increasing viscosity of the suspensions, which are exemplified by sodium carboxymethylcellulose, sorbitol and dextran. In addition, suspensions of the active components, such as oily injection suspension, include lipophilic solvents or carriers, which are exemplified by fatty oils such as sesame oil, and synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Polycationic non-lipid amino polymers may also be used as vehicles. Optionally, the suspensions may contain suitable stabilizers or drugs to increase the solubility of protein variants and obtain high concentrations of the protein variants.

The pharmaceutical composition of the present invention is preferably in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. Such suspension may be formulated according to the methods known in the art, using suitable dispersing or wetting agents (e.g., Tween 80) and suspending agents. The sterile injectable preparations may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, such as a solution in 1,3-butanediol. The acceptable vehicles and solvents include mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or di-glycerides. In addition, fatty acids, such as oleic acid and glyceride derivatives thereof, may be used in the preparation of injectable preparations, like the pharmaceutically acceptable natural oils (e.g., olive oil or castor oil), and particularly, polyoxyethylated derivatives thereof.

The aforementioned aqueous composition is sterilized mainly by filtration using a filter to remove bacteria, mixing with disinfectants or in combination with radiation. The sterilized composition can be hardened, for example, by freeze-drying to obtain a hardened product, and for

practical use, the hardened product is dissolved in sterilized water or a sterilized diluted solution.

The term "therapeutically effective amount", as used herein in connection with the pharmaceutical composition of the present invention, means an amount in which an active component shows an improved or therapeutic effect toward a disease to which the pharmaceutical composition of the present invention is applied. The therapeutically effective amount of the pharmaceutical composition of the present invention may vary according to the patient's age and sex, application sites, administration frequency, administration duration, formulation types and adjuvant types. Typically, the pharmaceutical composition of the present invention is administered in smaller amounts than a wild-type protein, for example, 0.01-1000 μg/kg/day, more preferably 0.1-500 μg/kg/day, and most preferably 1-100 μg/kg/day.

On the other hand, it will be apparent to those skilled in the art that diseases to which the present composition is applied may vary according to the protein type. The EPO and TPO altered as in an embodiment of the present invention may be used for treating, in addition to anemia itself, anemia as a complication associated with other diseases (e.g., anemia in inflammatory bowel disease, Progressive Kidney Disease, anemia of renal failure, the anemia associated with HIV infection in zidovudine (AZT) treated patients, anemia associated with cancer chemotherapy, Huntington's disease (HD), sickle cell anemia, Late Hyporegenerative Anemia in Neonates with Rh Hemolytic Disease after in utero Exchange Transfusion). In addition, the G-CSF altered according to the present invention may be used for treating neutropenia itself and neutropenia developed after bone marrow transplantation or cancer chemotherapy, the GH variants may be used for treating pituitary dwarfism and paediatric chronic renal failure. However, the present invention is not limited to these applications.

Hereinafter, the present invention provides interferon variants which each substitute valine for specific phenylalanine residue of 4-helix bundle cytokines, in detail, CNTF, EPO, Flt3L, G-CSF, GM-CSF, GH, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12p35, LPT, LIF, M-CSF, OSM, PL, SCF, TPO, IFN-α2A, IFN-α2B, IFN-β, IFN-γ, IFN-ω and IFN-τ.

In one specific aspect, the present invention provides the following protein variants: (1) a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119,

152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF; (2) an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO; (3) a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L; (4) a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence (SEQ ID NO.: 4) of a wild-type G-CSF; (5) a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF; (6) a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH; (7) an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A; (8) an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B; (9) an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β; (10) an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ; (11) an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω; (12) an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ; (13) an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2; (14) an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3; (15) an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4; (16) an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69,

96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5; (17) an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6; (18) an IL-12p35 variant that substitutes valine for the phenylalanine residue at the position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35; (19) a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT; (20) a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or 180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF; (21) a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF; (22) an OSM variant that substitutes valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM; (23) a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL; (24) a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF; and (25) a TPO variant that substitutes valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO.

In another specific aspect, the present invention provides the following DNA molecules: (1) a DNA encoding a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119, 152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF; (2) a DNA encoding an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO; (3) a DNA encoding a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L; (4) a DNA encoding a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence (SEQ ID NO.: 4) of a wild-type G-CSF; (5) a DNA encoding

a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF; (6) a DNA encoding a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH; (7) a DNA encoding an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A; (8) a DNA encoding an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B; (9) a DNA encoding an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β; (10) a DNA encoding an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ; (11) a DNA encoding an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω; (12) a DNA encoding an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ; (13) a DNA encoding an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2; (14) a DNA encoding an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3; (15) a DNA encoding an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4; (16) a DNA encoding an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69, 96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5; (17) a DNA encoding an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6; (18) a DNA encoding an IL-12p35 variant that substitutes valine for the phenylalanine residue at the

position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35; (19) a DNA encoding a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT; (20) a DNA encoding a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or 180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF; (21) a DNA encoding a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF; (22) a DNA encoding an OSM variant that substitutes valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM; (23) a DNA encoding a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL; (24) a DNA encoding a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF; and (25) a DNA encoding a TPO variant that substitutes valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO.

In a further specific aspect, the present invention provides the following recombinant expression vectors: (1) a recombinant expression vector to which a DNA encoding a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119, 152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF is operably linked; (2) a recombinant expression vector to which a DNA encoding an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO is operably linked; (3) a recombinant expression vector to which a DNA encoding a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L is operably linked; (4) a recombinant expression vector to which a DNA encoding a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence

(SEQ ID NO.: 4) of a wild-type G-CSF is operably linked; (5) a recombinant expression vector to which a DNA encoding a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF is operably linked; (6) a recombinant expression vector to which a DNA encoding a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH is operably linked; (7) a recombinant expression vector to which a DNA encoding an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A is operably linked; (8) a recombinant expression vector to which a DNA encoding an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B is operably linked; (9) a recombinant expression vector to which a DNA encoding an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β is operably linked; (10) a recombinant expression vector to which a DNA encoding an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ is operably linked; (11) a recombinant expression vector to which a DNA encoding an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω is operably linked; (12) a recombinant expression vector to which a DNA encoding an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ is operably linked; (13) a recombinant expression vector to which a DNA encoding an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2 is operably linked; (14) a recombinant expression vector to which a DNA encoding an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3 is operably linked; (15) a recombinant

expression vector to which a DNA encoding an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4 is operably linked; (16) a recombinant expression vector to which a DNA encoding an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69, 96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5 is operably linked; (17) a recombinant expression vector to which a DNA encoding an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6 is operably linked; (18) a recombinant expression vector to which a DNA encoding an IL-12p35 variant that substitutes valine for the phenylalanine residue at the position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35 is operably linked; (19) a recombinant expression vector to which a DNA encoding a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT is operably linked; (20) a recombinant expression vector to which a DNA encoding a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or 180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF is operably linked; (21) a recombinant expression vector to which a DNA encoding a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF is operably linked; (22) a recombinant expression vector to which a DNA encoding an OSM variant that substitutes valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM is operably linked; (23) a recombinant expression vector to which a DNA encoding a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL is operably linked; (24) a recombinant expression vector to which a DNA encoding a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF is operably linked; and (25) a recombinant expression vector to which a DNA encoding a TPO variant that substitutes

valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO is operably linked.

In yet another specific aspect, the present invention provides the following host cells: (1) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119, 152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF is operably linked; (2) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO is operably linked; (3) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L is operably linked; (4) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence (SEQ ID NO.: 4) of a wild-type G-CSF is operably linked; (5) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF is operably linked; (6) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH is operably linked; (7) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A is operably linked; (8) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B is operably linked; (9) a host cell

transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β is operably linked; (10) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ is operably linked; (11) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω is operably linked; (12) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ is operably linked; (13) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2 is operably linked; (14) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3 is operably linked; (15) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4 is operably linked; (16) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69, 96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5 is operably linked; (17) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6 is operably linked; (18) a host cell transformed or transfected

with a recombinant expression vector to which a DNA encoding an IL-12p35 variant that substitutes valine for the phenylalanine residue at the position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35 is operably linked; (19) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT is operably linked; (20) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or 180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF is operably linked; (21) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF is operably linked; (22) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an OSM variant that substitutes valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM is operably linked; (23) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL is operably linked; (24) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF is operably linked; and (25) a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a TPO variant that substitutes valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO is operably linked.

In still another specific aspect, the present invention provides the following methods of preparing a protein variant: (1) a method of preparing a protein variant, comprising cultivating a host

cell transformed or transfected with a recombinant expression vector to which a DNA encoding a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119, 152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF is operably linked, and isolating the protein variant from a resulting culture; (2) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO is operably linked, and isolating the protein variant from a resulting culture; (3) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L is operably linked, and isolating the protein variant from a resulting culture; (4) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence (SEQ ID NO.: 4) of a wild-type G-CSF is operably linked, and isolating the protein variant from a resulting culture; (5) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF is operably linked, and isolating the protein variant from a resulting culture; (6) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH is operably linked, and isolating the protein variant from a resulting culture; (7) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43,

47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A is operably linked, and isolating the protein variant from a resulting culture; (8) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B is operably linked, and isolating the protein variant from a resulting culture; (9) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β is operably linked, and isolating the protein variant from a resulting culture; (10) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ is operably linked, and isolating the protein variant from a resulting culture; (11) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω is operably linked, and isolating the protein variant from a resulting culture; (12) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ is operably linked, and isolating the protein variant from a resulting culture; (13) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2 is operably linked, and isolating the protein variant from a resulting culture; (14) a method

of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3 is operably linked, and isolating the protein variant from a resulting culture; (15) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4 is operably linked, and isolating the protein variant from a resulting culture; (16) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69, 96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5 is operably linked, and isolating the protein variant from a resulting culture; (17) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6 is operably linked, and isolating the protein variant from a resulting culture; (18) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an IL-12p35 variant that substitutes valine for the phenylalanine residue at the position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35 is operably linked, and isolating the protein variant from a resulting culture; (19) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT is operably linked, and isolating the protein variant from a resulting culture; (20) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or

180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF is operably linked, and isolating the protein variant from a resulting culture; (21) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF is operably linked, and isolating the protein variant from a resulting culture; (22) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding an OSM variant that substitutes valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM is operably linked, and isolating the protein variant from a resulting culture; (23) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL is operably linked, and isolating the protein variant from a resulting culture; (24) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF is operably linked, and isolating the protein variant from a resulting culture; and (25) a method of preparing a protein variant, comprising cultivating a host cell transformed or transfected with a recombinant expression vector to which a DNA encoding a TPO variant that substitutes valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO is operably linked, and isolating the protein variant from a resulting culture.

In still another specific aspect, the present invention provides the following pharmaceutical compositions: (1) a pharmaceutical composition comprising a CNTF variant that substitutes valine for the phenylalanine residue at the position 3, 83, 98, 105, 119, 152 or 178 of an amino acid sequence (SEQ ID NO.: 1) of a wild-type CNTF and a pharmaceutically acceptable carrier; (2) a

pharmaceutical composition comprising an EPO variant that substitutes valine for the phenylalanine residue at the position 48, 138, 142 or 148 of an amino acid sequence (SEQ ID NO.: 2) of a wild-type EPO and a pharmaceutically acceptable carrier; (3) a pharmaceutical composition comprising a Flt3L variant that substitutes valine for the phenylalanine residue at the position 6, 15, 81, 87, 96 or 124 of an amino acid sequence (SEQ ID NO.: 3) of a wild-type Flt3L and a pharmaceutically acceptable carrier; (4) a pharmaceutical composition comprising a G-CSF variant that substitutes valine for the phenylalanine residue at the position 13, 83, 113, 140, 144 or 160 of an amino acid sequence (SEQ ID NO.: 4) of a wild-type G-CSF and a pharmaceutically acceptable carrier; (5) a pharmaceutical composition comprising a GM-CSF variant that substitutes valine for the phenylalanine residue at the position 47, 103, 106, 113 or 119 of an amino acid sequence (SEQ ID NO.: 5) of a wild-type GM-CSF and a pharmaceutically acceptable carrier; (6) a pharmaceutical composition comprising a GH variant that substitutes valine for the phenylalanine residue at the position 1, 10, 25, 31, 44, 54, 92, 97, 139, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 6) of a wild-type GH and a pharmaceutically acceptable carrier; (7) a pharmaceutical composition comprising an IFN-α2A variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 7) of a wild-type IFN-α2A and a pharmaceutically acceptable carrier; (8) a pharmaceutical composition comprising an IFN-α2B variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 43, 47, 64, 67, 84, 123 or 151 of an amino acid sequence (SEQ ID NO.: 8) of a wild-type IFN-α2B and a pharmaceutically acceptable carrier; (9) a pharmaceutical composition comprising an IFN-β variant that substitutes valine for the phenylalanine residue at the position 8, 38, 50, 67, 70, 111 or 154 of an amino acid sequence (SEQ ID NO.: 9) of a wild-type IFN-β and a pharmaceutically acceptable carrier; (10) a pharmaceutical composition comprising an IFN-γ variant that substitutes valine for the phenylalanine residue at the position 18, 32, 55, 57, 60, 63, 84, 85, 95 or 139 of an amino acid sequence (SEQ ID NO.: 10) of a wild-type IFN-γ and a pharmaceutically acceptable carrier; (11) a pharmaceutical composition comprising an IFN-ω variant that substitutes valine for the phenylalanine residue at the position 27, 36, 38, 65, 68, 124 or 153 of an amino acid sequence (SEQ ID NO.: 11) of a wild-type IFN-ω and a pharmaceutically acceptable carrier; (12) a pharmaceutical

composition comprising an IFN-τ variant that substitutes valine for the phenylalanine residue at the position 8, 39, 68, 71, 88, 127, 156, 157, 159 or 183 of an amino acid sequence (SEQ ID NO.: 12) of a wild-type IFN-τ and a pharmaceutically acceptable carrier; (13) a pharmaceutical composition comprising an IL-2 variant that substitutes valine for the phenylalanine residue at the position 42, 44, 78, 103, 117 or 124 of an amino acid sequence (SEQ ID NO.: 13) of a wild-type IL-2 and a pharmaceutically acceptable carrier; (14) a pharmaceutical composition comprising an IL-3 variant that substitutes valine for the phenylalanine residue at the position 37, 61, 107, 113 or 133 of an amino acid sequence (SEQ ID NO.: 14) of a wild-type IL-3 and a pharmaceutically acceptable carrier; (15) a pharmaceutical composition comprising an IL-4 variant that substitutes valine for the phenylalanine residue at the position 33, 45, 55, 73, 82 or 112 of an amino acid sequence (SEQ ID NO.: 15) of a wild-type IL-4 and a pharmaceutically acceptable carrier; (16) a pharmaceutical composition comprising an IL-5 variant that substitutes valine for the phenylalanine residue at the position 49, 69, 96 or 103 of an amino acid sequence (SEQ ID NO.: 16) of a wild-type IL-5 and a pharmaceutically acceptable carrier; (17) a pharmaceutical composition comprising an IL-6 variant that substitutes valine for the phenylalanine residue at the position 73, 77, 93, 104, 124, 169 or 172 of an amino acid sequence (SEQ ID NO.: 17) of a wild-type IL-6 and a pharmaceutically acceptable carrier; (18) a pharmaceutical composition comprising an IL-12p35 variant that substitutes valine for the phenylalanine residue at the position 13, 39, 82, 96, 116, 132, 150, 166 or 180 of an amino acid sequence (SEQ ID NO.: 18) of a wild-type IL-12p35 and a pharmaceutically acceptable carrier; (19) a pharmaceutical composition comprising a LPT variant that substitutes valine for the phenylalanine residue at the position 41 or 92 of an amino acid sequence (SEQ ID NO.: 19) of a wild-type LPT and a pharmaceutically acceptable carrier; (20) a pharmaceutical composition comprising a LIF variant that substitutes valine for the phenylalanine residue at the position 41, 52, 67, 70, 156 or 180 of an amino acid sequence (SEQ ID NO.: 20) of a wild-type LIF and a pharmaceutically acceptable carrier; (21) a pharmaceutical composition comprising a M-CSF variant that substitutes valine for the phenylalanine residue at the position 35, 37, 54, 67, 91, 106, 121, 135, 143, 229, 255, 311, 439, 466 or 485 of an amino acid sequence (SEQ ID NO.: 21) of a wild-type M-CSF and a pharmaceutically acceptable carrier; (22) a pharmaceutical composition comprising an OSM variant that substitutes

valine for the phenylalanine residue at the position 56, 70, 160, 169, 176 or 184 of an amino acid sequence (SEQ ID NO.: 22) of a wild-type OSM and a pharmaceutically acceptable carrier; (23) a pharmaceutical composition comprising a PL variant that substitutes valine for the phenylalanine residue at the position 10, 31, 44, 52, 54, 92, 97, 146, 166, 176 or 191 of an amino acid sequence (SEQ ID NO.: 23) of a wild-type PL and a pharmaceutically acceptable carrier; (24) a pharmaceutical composition comprising a SCF variant that substitutes valine for the phenylalanine residue at the position 63, 102, 110, 115, 116, 119, 126, 129, 158, 199, 205, 207 or 245 of an amino acid sequence (SEQ ID NO.: 24) of a wild-type SCF and a pharmaceutically acceptable carrier; and (25) a pharmaceutical composition comprising a TPO variant that substitutes valine for the phenylalanine residue at the position 46, 128, 131, 141, 186, 204, 240 or 286 of an amino acid sequence (SEQ ID NO.: 25) of a wild-type TPO and a pharmaceutically acceptable carrier.

The present purpose to improve the efficacy in modulating biological responses was accomplished in the following examples using TPO, EPO, G-CSF and GH. It will be apparent to those skilled in the art that the following examples are provided only to illustrate the present invention, and the scope of the present invention is not limited to the examples.

Example 1. Construction of DNA coding wild type TPO/EPO/G-CSF/GH

A. Construction of DNA coding wild type TPO

750㎕ of TRIzol reagent(MRC.,USA) was added to bone marrow tissue in a microcentrifuge tube and incubated at room temperature for 5 minutes. 200㎕ of chloroform was added into the tube and then the tube was shaken vigorously for 15 seconds. After incubating the tube at room temperature for 2-3 minutes, it was centrifuged at 15,000rpm for 15 minutes at 4℃. The upper phase was transferred to a 1.5㎖ tube and 500㎕ of isopropanol was added. The sample was incubated at -70℃ for 30 minutes and centrifuged at 15,000rpm for 15 minutes at 4℃. After discarding supernatant, RNA pellet was washed once with 75% DEPC-ethanol by vortexing and centrifuged at 15,000rpm for 15 minutes at 4℃. The supernatant was removed and the RNA pellet

was dried for 5 minutes at room temperature and then the pellet was dissolved in 50㎕ of DEPC-treated 3° distilled water.

2㎍ of mRNA purified as above and 1㎕ of oligo dT30 primer(10 μM, Promega, USA) were mixed and heated at 70℃ for 2 minutes and then it was immediately cooled on ice for 2 minutes. After that, this reaction mixture was added with 200U M-MLV reverse transcriptase(Promega, USA), 10㎕ of 5X reaction buffer(250mM Tris-HCl, pH 8.3, 375mM KCl, 15mM $MgCl_2$, 50nM DTT), 1㎕ of dNTP(10mM dATP, 10mM dTTP, 10mM dGTP, 10mM dCTP) and DEPC-treated 3° water was added to make the total volume of 50㎕. After mixing gently, the reaction mixture was incubated at 42℃ for 60 minutes.

To amplify cDNA coding wild type TPO, the first strand cDNA as template, primer 1 and primer 2 (Table 1) were added into a PCR tube including 2U of *pfu* DNA polymerase(Stratagene, USA), 10㎕ of 10X reaction buffer, 1% Triton X-100, 1mg/ml BSA, 3㎕ of primer 1(10 μM), 3㎕ of primer 2(10 μM), 2㎕ of dNTP(10mM dATP, 10mM dTTP, 10mM dGTP, 10mM dCTP), and distilled water was added to make the total volume of 100㎕. The PCR reaction condition was as follows; 1 cycle at 95℃ for 3 minutes, and then 30 cycles at 95℃ for 30 seconds, at 52℃ for 1 minute, and at 72℃ for 1.5 minutes, and finally 1 cycle at 72℃ for 10 minutes to make PCR product with completely blunt end.

The PCR product obtained was separated in 0.8% agarose gel(BMA, USA) and was purified with Qiaex Ⅱ gel extraction kit (Qiagen, USA). After the isolated DNA was mixed with 15U of *EcoR*I, 10U of *Not*I , 3㎕ of 10X reaction buffer and 3° distilled water was added to make the total volume of 30㎕, DNA was restricted by incubation at 37℃ for 2 hours. The PCR product was separated in 0.8% agarose gel and was purified with Qiaex Ⅱ gel extraction kit.

After 5㎍ of pBluescript KS Ⅱ(+) vector was mixed with 15U of *EcoR*I, 10U of *Not*I , 3㎕ of 10X reaction buffer and 3° distilled water was added to make the total volume of 30㎕, DNA was restricted by incubation at 37℃ for 2 hours. The restricted pBluescript KS Ⅱ(+) vector was separated in 0.8% agarose gel and was purified with Qiaex Ⅱ gel extraction kit.

100ng of the digested pBluescript KS Ⅱ(+) vector was ligated with 20ng of the PCR product which was digested with same enzymes. This ligation mixture was incubated at 16℃ water bath for

16 hours, thus producing a recombinant vector comprising cDNA coding wild type TPO. Then, it was transformed into a *E.coli* Top10(Invitrogen, USA) which was made to a competent cell by rubidium chloride method. The transformed bacteria was cultured on LB agar plate containing 50㎍/㎖ of ampicillin(Sigma, USA). After overnight incubation, colonies were transferred into tubes with 3㎖ of LB medium containing 50㎍/㎖ ampicillin and then they were cultured at 37℃ for 16 hours. Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of *EcoRI/NotI* was used to detect inclusion of cloned gene in the plasmid.

B. Construction of DNA coding wild type EPO

Procedure of cloning DNA coding wild type EPO was basically same to that used for cloning DNA coding wild type TPO.

The first strand cDNA as template, primer 11 and primer 12 (Table2) were used for PCR amplification of DNA coding wild type EPO. The PCR product and cloning vector, pBluescript KS Ⅱ(+) were digested with both *EcoR*I and *BamH*I endonucleases. The digested PCR product and cloning vector were ligated and transformed into competent cell, *E.coli* Top10(Invitrogen, USA). Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of *EcoRI/BamH*I was used to detect existence of cloned gene in the plasmid.

C. Construction of DNA coding wild type G-CSF

Construction procedure of DNA coding wild type G-CSF was similar to that used for DNA coding wild type TPO.

Leukocytes from healthy people were used for the mRNA extraction, and primers 21 and 22 (Table 3) were used for PCR amplification of cDNA coding wild type G-CSF. Both the PCR product and cloning vector, pBluescript KS Ⅱ(+) were digested with *Sma*I and *EcoR*I endonuclease. The digested PCR product and cloning vector were ligated and transformed into competent cell, *E.coli* Top10(Invitrogen, USA). Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of *SmaI/EcoR*I was used to detect existence of cloned gene in the plasmid.

D. Construction of DNA coding wild type GH

DNA coding wild type GH was purchased from ATCC(ATCC No. 67097). To add leader sequence to N-terminal end of this cDNA, primer 35 and 36 (Table 4) were used for PCR. In order to make complete cDNA coding wild type GH linked to the leader sequence, secondary PCR was carried out using primers 37 and 38 (Table 4). The PCR product and cloning vector, pBluescript KS II(+) were digested with *EcoR*I and *Hind*III endonuclease. Plasmid was isolated from the cultured bacteria with alkaline lysis method and the restriction of *EcoRI*/*Hind*III was used to detect existence of cloned gene in the plasmid.

Example 2. Construction of cDNA coding TPO/EPO/G-CSF/GH muteins

A. Construction of cDNAs coding TPO muteins

Four muteins of TPO, TPO-[F46V], TPO-[F128V], TPO-[F131V] and TPO-[F141V] were constructed according to procedures as follows to have a single amino acid-substitution from phenylalanine to valine at each positions, respectively.

<Table 1>

Primers used in constructing cDNAs coding TPO- wild type and muteins

Primer No.			Nucleotide sequence	Sequence No.
1	Wild type TPO	Sense	5'-CGGAATTCCGATGGAGCTGACTGAATTG-3'	26
2		Antisense	5'-TTTAGCGGCCGCATTCTTACCCTTCCTGAG-3'	27
3	TPO-[F46V]	Sense	T3	
4		Antisense	5'-CCAAGCTAACGTCCACAGCAG-3'	28
5	TPO-[F128V]	Sense	T3	
6		antisense	5'-GCTCAGGACGATGGCAT-3'	29
7	TPO-[F131V]	Sense	T3	
8		antisense	5'-GGTGTTGGACGCTCAGGAAGATG-3'	30
9	TPO-[F141V]	Sense	T3	
10		antisense	5'-CATCAGGACACGCACCTTTCC-3'	31

cDNA which code TPO-[F46V], TPO-[F128V], TPO-[F131V] and TPO-[F141V] was constructed by primary PCR using specific primers (Table 1) and universal primer T3 and secondary

PCR using the primary PCR product and universal primer T7. The template for these reactions was the cDNA coding wild type TPO cloned in pBluescript KS II(+) obtained from Example 1.

The primary PCR was performed by adding 2.5U Ex taq(Takara, Japan), 5㎕ of 10X buffer, 1mM $MgCl_2$, 2.5mM dNTP and D.W was added to make the total volume of 50㎕. The PCR condition consisted of 1 cycle at 94℃ for 3 minutes followed by 30 cycles at 95℃ for 30 seconds, at 60℃ for 30 seconds and at 72℃ for 30 seconds and then linked to 1 cycle at 72℃ for 7 minutes. The primary PCR product was used as a megaprimer in the secondary PCR together with universal primer T7(10pmole). The cDNA coding wild type TPO cloned in pBluescript KS II(+) was used as the template in the secondary PCR. The secondary PCR was performed by adding 2.5U Ex taq, 5㎕ of 10X buffer, 2.5mM dNTP and D.W was added to make the total volume of 50㎕. The PCR condition consisted of 1 cycle at 94℃ for 3 minutes followed by 30 cycles at 94℃ for 1 minute, at 58℃ for 1 minute, and at 72℃ for 1.5 minutes and finally linked to 1 cycle at 72℃ for 7 minutes prior to termination.

To minimize errors derived form DNA synthesis, Mg^{2+} concentration was reduced to 1mM in the primary PCR. Sizes of megaprimers amplified were 280b.p for TPO-[F46V], 520b.p for TPO-[F128V], 530b.p for TPO-[F131V] and 560b.p for TPO-[F141V]. In the secondary PCR using megaprimers, cDNA coding each muteins produced showed the same size of 1062b.p. Substitution from phenylalanine to valine at nucleotide sequence of the individual TPO mutein was verified by direct sequencing.

Each PCR product of 1062b.p was separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit. The PCT product was digested with 15U *EcoR*I and 10U *Not*I at 37°C for 2 hours. The digested PCR product was separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit and ligated with pBluescript KS II(+) as described above. The recombinant expression vector containing DNA which codes TPO-[F141V] was named Tefficacin-4 and was deposited at the KCCM(Korean Culture Center of Microorganisms) under the Budapest Treaty on June 9, 2003. Accession number given by international depositary authority was KCCM-10500.

B. Construction of cDNAs coding EPO muteins

Four muteins of EPO, EPO-[F48V], EPO-[F138V], EPO-[F142V] and EPO-[F148V] were constructed according to procedures as follows to have a single amino acid-substitution from phenylalanine to valine at each positions, respectively.

<Table 2>

Primers used in constructing cDNAs coding EPO- wild type and muteins

Primer No.			Nucleotide sequence	Sequence No.
11	Wild EPO	Sense	5'-GGCGCGGAGATGGGGGT-3'	32
12		Antisense	5'-TGGTCATCTGTCCCCTGTCCTG-3'	33
13	EPO-[F48V]	Sense	T3	
14		Antisense	5'-GACATTAACTTTGGTGTCTGGGAC-3'	34
15	EPO-[F138V]	Sense	5'-CTGTCCGCAAACTCTTCCGAG-3'	35
16		Antisense	T7	
17	EPO-[F142V]	Sense	5'-CGCAAACTCGTCCGAGTCTACT-3'	36
18		Antisense	T7	
19	EPO-[F148V]	Sense	5'-GAGTCTACTCCAATGTGGTGGG-3'	37
20		Antisense	T7	

Construction procedure of cDNA coding EPO muteins was basically similar to that of TPOs. cDNAs which code EPO-[F48V], EPO-[F138V], EPO-[F142V], and EPO-[F148V] were constructed by primary PCR using specific primers (Table 2) and universal primer T3 and secondary PCR using the primary PCR product and universal primer T7. The template for these reactions was the cDNA coding wild type EPO cloned in pBluescript KS II(+) obtained from Example 1.

Mg^{2+} concentration was adjusted to 1mM in the primary PCR. Sizes of amplified megaprimers were 300b.p for EPO-[F48V], 550b.p for EPO-[F138V], 550b.p for EPO-[F142V] and 550b.p for EPO-[F148V]. In the secondary PCR using the megaprimers, cDNAs coding the individual muteins were amplified as the same size of 580b.p. Substitution from phenylalanine to valine at nucleotide sequence of the individual EPO mutein was verified by direct sequencing.

Each PCR product of 580b.p was separated in 0.8% agarose gel and was purified with Qiaex II gel extraction kit. The PCR product was digested with 15U *EcoR*I and 10U *BamH* I at 37°C for 2 hours. The digested PCR product was ligated into pBluescript KS II(+) as described above and was used for constructing the expression vector. The recombinant expression vector containing DNA

which codes TPO-[F141V] was named Refficacin-4 and was deposited at the KCCM(Korean Culture Center of Microorganisms) under the Budapest Treaty on June 9, 2003. Accession number given by international depositary authority was KCCM-10501.

C. Construction of cDNAs coding G-CSF muteins

Muteins of G-CSF, G-CSF[F13V], G-CSF[F83V], G-CSF[F113V], G-CSF[F140V], G-CSF[F144V] and G-CSF[F160V] were constructed according to procedures as follows to have a single amino acid-substitution from phenylalanine to valine at each positions, respectively.

<Table 3>

Primers used in constructing cDNAs coding G-CSF- wild type and muteins

Primer No.			Nucleotide sequence	Sequence No.
21	wild G-CSF	Sense	5'-CCCCGGGACCATGGCTGGACCTGCCACCCAG-3'	38
22		Antisense	5'-CGAATTCGCTCAGGGCTGGGCAAGGAG-3'	39
23	G-CSF-[F13V]	Sense	T7	
24		Antisense	5'-ACTTGAGCAGGACGCTCT-3'	40
25	G-CSF-[F83V]	Sense	5'-AGCGGCCTTGTCCTCTA-3'	41
26		Antisense	T3	
27	G-CSF-[F113V]	Sense	5'-GACGTTGCCACCACCAT-3'	42
28		Antisense	T3	
29	G-CSF-[F140V]	Sense	5'-GCCGTCGCCTCTGCTTT-3'	43
30		Antisense	T3	
31	G-CSF-[F144V]	Sense	5'-TCGCCTTCTGCTGTCCAG-3'	44
32		Antisense	T3	
33	G-CSF-[F160V]	Sense	5'-TCTGCAAGACGTCCTGG-3'	45
34		Antisense	T3	

Construction procedure of cDNA coding G-CSF muteins was basically similar to that of TPOs. cDNAs which code G-CSF-[F13V], G-CSF-[F83V], G-CSF-[F113V], G-CSF-[F140V], G-CSF-[F144V], and G-CSF-[F160V] were constructed by primary PCR using specific primers (Table 3) and universal primer T3 and secondary PCR using the primary PCR product and universal primer T7. The template for these reactions was the cDNA coding wild type G-CSF cloned in pBluescript KS II(+) obtained from the Example 1.

Mg^{2+} concentration was adjusted to 1mM in the primary PCR. Sizes of amplified megaprimers were 600b.p for G-CSF-[F13V], 390b.p for G-CSF-[F83V], 300b.p for G-CSF-[F113V],

200b.p for G-CSF-[F140V], 200b.p for G-CSF-[F144V], and 150 b.p for G-CSF[F160V]. In the secondary PCR using the megaprimers, cDNAs coding each muteins were amplified as the same size of 640b.p. Substitution from phenylalanine to valine at nucleotide sequence of the individual G-CSF mutein was verified by direct sequencing.

Each PCR product of 640b.p was separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit. The PCR product was digested with 15U *Sma*I and 10U *Eco*RI at 37°C for 2 hours and separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit. The digested PCR product was ligated into pBluescript KS II(+) as described above. The recombinant expression vector containing DNA which codes G-CSF-[F140V] was named Grefficacin4 and was deposited at the KCCM(Korean Culture Center of Microorganisms) under the Budapest Treaty on May 17, 2004. Accession number given by international depositary authority was KCCM-10571.

D. Construction of cDNAs coding GH muteins

Four muteins of GH, GH-[F44V], GH-[F97V], GH-[F139V], GH-[F146V], GH-[F166V], and GH-[F176V] were constructed according to procedures as follows to have a single amino acid-substitution from phenylalanine to valine at each positions, respectively.

<Table 4>

Primers used in constructing cDNAs coding GH- wild type and muteins

Primer No.			Nucleotide Sequence	Sequence No.
35	Leader sequence addition	Sense-1	5'-CTTTTGGCCTGCTCTGCCTGTCCTGGCTTCAA GAGGGCAGTGCCTTCCCAACCATTCCCTTATC-3'	46
36		Antisense	T3	
37		Sense-2	5'-GGAATTCATGGCTGCAGGCTCCCGGACGTCC CTGCTCCTGGCTTTTGGCCTGCTCTGCCT-3'	47
38		Antisense	T3	
39	GH-[F44V]	Sense	T7	
40		Antisense	5'-GGGGTTCTGCAGGACTGAATACTTC-3'	48
41	GH-[F97V]	Sense	T7	
42		Antisense	5'-GGCTGTTGGCGACGATCCTG-3'	49
43	GH-[F139V]	Sense	T7	
44		Antisense	5'-GTAGGTCTGCTTGACGATCTGCCCAG-3'	50
45	GH-[F146V]	Sense	T7	
46		Antisense	5'-GAGTTTGTGTCGACCTTGCTGTAG-3'	51
47	GH-[F166V]	Sense	T7	
48		Antisense	5'-GTCCTTCCTGACGCAGTAGAGCAG-3'	52
49	GH-[F176V]	Sense	T7	
50		Antisense	5'-CGATGCGCAGGACTGTCTCGACCTTGTC-3'	53

Construction procedure of cDNA coding GH muteins was basically similar to that of TPOs. cDNAs which code muteins GH-[F44V], GH-[F97V], GH-[F139V], GH-[F146V], GH-[F166V] and GH-[F176V] were constructed by primary PCR using specific primers (Table 4) and universal primer T3 and secondary PCR using the primary PCR product and universal primer T7. The template for these reactions was the cDNA coding wild type GH cloned in pBluescript KS II(+) obtained from Example 1.

Mg^{2+} concentration was adjusted to 1mM in the primary PCR. Sizes of each amplified megaprimers were 130b.p for GH-[F44V], 300b.p for GH-[F97V], 420b.p for GH-[F139V], 450b.p for GH-[F146V], 500b.p for GH-[F166V] and 530b.p for GH-[F176V] PCRs. Substitution from phenylalanine to valine at nucleotide sequence of the individual GH mutein was verified by direct sequencing.

Each PCR product of 650b.p was separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit. The PCR product was digested with 15U *EcoR*I and 10U *Hind*III at 37°C for 2

hours and separated in 0.8% agarose gel and purified with Qiaex II gel extraction kit. The digested PCR product was ligated into pBluescript KS II(+) as described above.

EXAMPLE 3. Expression and Purification of TPO muteins

A. TPO muteins

a. Establishments of transfected cell lines by using Lipofection method

Chinese hamster ovary ("CHO-K1")(ATCC, CCL61) cells were prepared at a density 1.5×10^5 cells per 35mm dish containing Dulbecco's modified Eagle's medium ("DMEM")[Gibco BRL, USA] supplemented with 10% fetal bovine serum("FBS"). The cells were grown at 37℃ in a 5% CO_2 for 18-24 hrs. 6㎕ of Lipofectamine was added to 1.5㎍ of the recombinant expression vector comprising DNA coding TPO mutein in a sterile tube. Volume of this mixture was adjusted to 100㎕ by adding serum-free DMEM. The tube was incubated at room temperature for 45 min. The cells grown in 35mm dish were washed twice with serum-free DMEM and 800㎕ of serum-free DMEM was added to the dish. The washed cells were gently overlaid on the lipofectamine-DNA complex and then incubated for 5hrs at 37℃ in 5% CO_2. After 5 hrs incubation, 1㎖ of DMEM containing 20% FBS was added to transfected cells and then the cells were incubated for 18-24 hrs at 37℃, 5% CO_2. After the incubation, the cells were washed twice with serum-free DMEM and then 2㎖ of DMEM containing 10% FBS was added to the culture. These cells were incubated for 72 hrs at 37℃, 5% CO_2.

b. Analysis of expression level of TPO muteins using ELISA

The cells transfected with plasmid containing cDNA coding TPO-wild type or muteins were analyzed on their protein expression level by using ELISA assay. An goat anti-human TPO polyclonal antibody(R&D, U.S.A) diluted to 10㎍/㎖ with coating buffer[0.1M Sodium bicarbonate,(pH 9.6)] was added into each wells of 96 well plate(Falcon, USA) up to 100㎕ per well and incubated for 1 hour at room temperature. The plate was washed with 0.1% Tween-20 in 1X PBS(PBST) three times. After washing, the plate was incubated with 200㎕ of blocking buffer(1%

FBS, 5% sucrose, 0.05% sodium azide) for 1 hour at room temperature and then washed three times with PBST. The cultured supernatants (including the transfected cells) and dilution buffer[0.1% BSA, 0.05% Tween-20, 1X PBS] were mixed with serial dilutions. 25ng/ml of recombinant human TPO[Calbiochem, USA] as a positive control and untransfected CHO-K1 cultured supernatants as a negative control were equally diluted. These controls and samples were incubated for 1 hr at room temperature. Then, the plate was washed with PBST three times. A biotinylated goat anti-human TPO antibody (R&D, USA) diluted to 0.2㎍/㎖ with dilution buffer was added to the 96 well plate up to 100㎕ per well and incubated for 1 hr at room temperature. The plate was washed with PBST three times. Streptavidin-HRP (R&D, USA) diluted to 1:200 in dilution buffer was added 100㎕ per well to the 96 well plate and incubated for 1 hr at room temperature. After 1 hour, the plates was washed three times with PBST, and then coloring reaction was performed by using TMB microwell peroxidase substrate system(KPL, USA) and O.D was read at 630nm with microplate reader[BIO-RAD, Model 550].

c. Analysis of expression level and molecular weight of mutein TPO using western blotting

In order to exclude FBS in medium, CHO-S-SFM II (Gibco BRL, USA) was used for culture of the above-transfected cell. Culture medium from CHO-S-SFM II was filtrated with 0.2㎛ syringe filter and concentrated with centricon (Mol. 30,000 Millipore, USA). To perform the reduced SDS-PAGE, sample-loading buffer containing 5% β-mercaptoethanol was added to the sample and heated for 5 minutes. Stacking gel and running gel were used for this SDS-PAGE. The stacking gel was composed of 3.5% acrylamide, 0.375 M Tris (pH6.8), 0.4% SDS and the running gel was composed of 10% acrylamide gel, 1.5 M Tris (pH8.8), 0.4% SDS. After SDS-PAGE gel running treatment, protein samples were transferred to Westran (PVDF transfermembrane, S&S) having 4㎛ pore at 350mA for 2 hrs in a 25mM Tris-192mM glycine (pH 8.3) -20% methanol buffer-containing reservoir. After transferring, it was blocked three times for 10 minutes with 5% fat free milk powder in PBST. The biotinylated goat anti-human TPO antibody (R&D, USA) was diluted to 0.25㎍/㎖ in blocking buffer and 3ml of this solution was added and shaken for 6 hrs. The membrane was washed with washing solution three times. Streptavidin-HRP (R&D, USA) was diluted to 1:100 in blocking buffer and

incubated for 1 hr. The membrane was washed three times with washing solution. Protein bands were visualized by incubating with DAB substrate (VECTOR LABORATORIES, USA) for 10 minutes. This reaction was stopped with soaking the membrane in deionized water.

In Figure 2a, wild type and mutein forms of TPOs had the same molecular weight(55kD).

Relative expression level of wild type and muteins of TPO was shown in Figure 3a. Expression level of each TPO mutein was compared to that of wild type TPO as a control. Expression level of TPO-[F128V] was increased 1.4 times more than that of wild type TPO. But expressions of TPO -[F46V], -[F131V] and -[F141V] were decreased to 20%, 40%, and 40% of that of wild type, respectively.

B. EPO muteins

Expression vectors containing cDNAs coding EPO muteins were transfected to CHO-K1 cell and expression level of each of EPO mutein was detected by using ELISA assay. And molecular weight of each of wild type and mutein of EPO was analyzed by western blotting.

In Figure 2b, wild type and mutein forms of EPO had the same molecular weight (45kD).

Relative expression level of wild type and muteins of EPO was shown in Figure 3b. Expressions level of EPO-[F48V] and -[F138V] was increased 1.4 and 1.2 times more than that of the wild type EPO, respectively. But expression level of EPO -[F142V] and -[F148V] was decreased to 20 % and 30 % of that of wild type EPO, respectively.

C. G-CSF muteins

Expression vectors containing cDNAs coding G-CSF muteins were transfected to CHO-K1 cell and expression level of each G-CSF mutein was detected by using ELISA assay. And molecular weight of each of wild type and muteins of G-CSF was analyzed by western blotting.

In Figure 2c, wild type and mutein forms of G-CSF had the same molecular weight (50kD).

Relative expression level of wild type- and muteins of G-CSF was shown in Figure 3c. Expression levels of rest of G-CSF muteins were similar to that of wild type G-CSF. Expression level of G-CSF mutein-[F83V] was increased 1.9 times than that of wild-type. But expression levels of G-

CSF muteins -[F140V] and -[F144V] were decreased to 50 % and 70 % of that of wild type G-CSF, respectively.

D. GH muteins

Expression vectors containing cDNAs coding GH muteins were transfected to CHO-K1 cell. Method for the expression of each of the GH muteins was the same as those used for TPO production.

EXAMPLE 4. Construction of DNA coding EPO, TPO, G-CSF, and GH receptors

A. Construction of DNA coding EPO and TPO receptors

DNAs coding EPO and TPO receptors were constructed to analyze binding affinities of each of EPO muteins and TPO muteins. DNA coding extracellular domain of each receptor was linked to DNA coding Fc domain of IgG1 such that the C-terminal region of extracellular domain of each receptor was fused to N-terminal region of human IgG1 Fc domain. cDNA coding EPO receptor was constructed by PCR using sense primer(primer 51) with restriction sites of *EcoR*I and leader sequence of EPO receptor and antisense primer(primer 52) with the sequence coding 3' end of EPO receptor and the sequence coding 5'end Fc domain of IgG. cDNA coding TPO receptor linked to Fc domain of IgG1 was constructed by PCR using sense primer(primer 53) with restriction sites of *Hind*III and leader sequence of TPO receptor and antisense primer(primer 54) with the sequence coding 3' end of TPO receptor and the sequence coding 5'end of Fc domain of IgG.

cDNA coding EPO receptor produced as described above and DNA coding Fc domain of IgG1 were mixed in the same tube, complementary binding between the common sequences was induced. Using this mixture, cDNA coding EPO receptor linked to Fc domain of IgG1 was constructed by PCR using sense primer(primer 51) with restriction sites of *EcoR*I and leader sequence of EPO receptor and antisense primer(primer 55) with restriction sites of *Xba*I and 3'end of Fc domain of IgG. The PCR product was cut with *EcoR*I and *Xba*I and inserted into PCR-3 expression vector for production of EPO receptor-Fc fusion protein.

cDNA coding TPO receptor produced as described above and DNA coding Fc domain of IgG1 were mixed in the same tube, thus complementary binding between the common sequences was induced. Using this mixture, cDNA coding TPO receptor linked to Fc domain of IgG1 was constructed by PCR using sense primer(primer 53) with restriction sites of *EcoR*I and leader sequence of EPO receptor and antisense primer(primer 55) with restriction sites of *Xba*I and 3'end of Fc domain of IgG. The PCR product was cut with *Hind*III and *Xba*I and inserted into PCR-3 expression vector for production of TPO receptor-Fc fusion protein.

<Table 5>

A List of primers used in constructing TPO and EPO receptors fused to immunoglobulin

	Primer No.		Nucleotide sequence	Sequence No.
EPO receptor	51	Sense	5'-CGGAATTCATGGACCACCTCGGGGCG-3'	54
	52	Antisense	5'-GCTCTAGACTAAGAGCAAGCCACATAGCTGGG-3'	55
TPO receptor	53	Sense	5'-CCCAAGCTTATGGAGCTGACTGAATTGCTCCTC-3'	56
	54	Antisense	5'-GGAATTCTTACCCTTCCTGAGACAGATTCTGG-3'	57
IgG1-R-XbaI	55		5'-GCTCTAGAGCTCATTTACCCGGAGACAGGGAGAG-3'	58

B. Construction of DNA coding G-CSF and GH receptors

cDNA coding G-CSF receptor was constructed by PCR using sense primer(primer 56) with restriction site of *Hind*III and leader sequence of G-CSF receptor and antisense primer(primer 57) with restriction site of *EcoR*I and the sequence coding 3' end of G-CSF receptor. cDNA coding GH receptor was constructed by PCR using sense primer (primer 58) with restriction site of *EcoR*I and leader sequence of G-CSF receptor and antisense primer(primer 59) with restriction site of *Spe*I and the sequence coding 3' end of G-CSF receptor.

The PCR product encoding G-CSF receptor was digested with *Hind*III and *EcoR*I, and was cloned by inserting into a commercially available cloning vector, pBluescript KS II(+) at *Hind*III/*EcoR*I site. The PCR product encoding GH receptor was digested with *EcoR*I and *Spe*I, and cloned by inserting into a commercially available cloning vector, pBluescript KS II(+) at *EcoR*I/ *Spe*I site.

Fc domain of human IgG was constructed by PCR using sense primer(primer 60 for G-CSF, primer 61 for GH) with sequence coding 5' end part of hinge region of human IgG and antisense primer (primer 62). For G-CSF receptor, the PCR product coding Fc domain of human IgG was digested with *EcoR*I and *Xba*I, and cloned by inserting into a commercially available cloning vector, pBluescript KS Ⅱ(+) at *EcoR*I/ *Xba*I site. For GH receptor, the PCR product coding Fc domain of human IgG was digested with *Spe*I and *Xba*I, and cloned by inserting into a commercially available cloning vector, pBluescript KS Ⅱ(+) at *Spe*I site/ *Xba*I.

Both of the cloned cDNA coding G-CSF receptor and the cloned Fc domain of human IgG were digested with *EcoR*I/ *Xba*I and then ligated to prepare DNA coding G-CSF receptor linked to Fc domain of human IgG. This DNA construct was cut with *Hind*III and *Xba*I and inserted into PCR-3 expression vector. Both of the cloned cDNA coding GH receptor and the cloned Fc domain of human IgG were digested with *Spe*I/ *Xba*I and then ligated to prepare DNA coding G-CSF receptor linked to Fc domain of human IgG. This DNA construct was cut with *EcoR*I and *Xba*I and inserted into PCR-3 expression vector.

<Table 6>

A List of primers used in constructing G-CSF and GH receptors fused to Immunoglobulin

	Primer No.		Nucleotide sequence	Sequence No.
G-CSF receptor	56	Sense	5'-CCCAAGCTTATGGCTGGACCTGCCACCC-3'	59
	57	Antisense	5'-GGAATTCGCAACAGAGCCAGGCAGTTCCA-3'	60
GH receptor	58	Sense	5'-CGGAATTCATGGATCTCTGGCAGCTG-3'	61
	59	Antisense	5'-GGACTAGTTTGGCTCATCTGAGGAAGTG-3'	62
IgG1-F-EcoR I	60	Sense	5'-GGAATTCGCAGAGCCCAAATCTTGTGACAAAACTC-3'	63
IgG1-F-Spe I	61	Sense	5'-GACTAGTGCAGAGCCCAAATCTTGTGA-3'	64
IgG1-R-XbaI	62	Antisense	5'-GCTCTAGAGCTCATTTACCCGGAGACAGGGAGAG-3'	65

EXAMPLE 5. Measurement of binding affinity of cytokines and their muteins to each of their receptors by using ELISA

A. Binding of TPO and TPO muteins to TPO Receptor

Culture supernatants of CHO cell transfected with expression vectors carrying genes for TPO muteins were used for measuring cytokine-receptor interactions.

TPO receptor-Ig fusion protein was purified from culture supernatant of CHO cell transfected with recombinant expression vector carrying gene coding for TPO receptor-Fc fusion protein by using Protein A Sepharose-4B column (Pharmacia, Sweden). The purified fusion protein diluted to 10$\mu g/m\ell$ with coating buffer [0.1M Sodium bicarbonate,(pH 9.6)] was added into each wells of 96 well plate(Falcon, USA) up to 100$\mu\ell$ per well and incubated for 1 hour at room temperature. The plate was washed with 0.1% Tween-20 in 1X PBS[PBST] three times. After washing, the plate was incubated with 200$\mu\ell$ of blocking buffer(1% FBS, 5% sucrose, 0.05% sodium azide) for 1 hour at room temperature and then washed three times with PBST.

After washing, culture supernatants consisting of four TPO muteins and one TPO wild type, respectively were diluted serially with dilution buffer[0.1% BSA, 0.05% Tween-20, 1X PBS] and was added to 96 well plate coated with the TPO receptor-Fc fusion protein and incubated for 1 hr. The washing was repeated three times with PBST. A recombinant human TPO[Calbiochem, USA] as a positive control, and untransfected CHO-K1 cultured supernatants as a negative control were equally diluted. The plates were washed with PBST three times. A biotinylated goat anti-human TPO antibody (R&D, USA) diluted to 0.2$\mu g/m\ell$ in dilution buffer was added to the 96 well plate to 100$\mu\ell$ per well and incubated for 1 hr at room temperature. The plate was washed with PBST three times. Streptavidin-HRP (R&D, USA) diluted to 1:200 in dilution buffer was added 100$\mu\ell$ per well to 96 well plate and incubated for 1 hr at room temperature. The plate was washed three times with PBST after 1 hour. Coloring reaction was performed using TMB microwell peroxidase substrate system (KPL, USA) and O.D was read at 630nm with microplate reader [BIO-RAD, Model 550].

The binding affinity of TPO-[F141V] and TPO-[F131V] to the TPO receptor was increased compared to that of wild type TPO (Figure 4a). And the former mutein had the strongest binding affinity among all TPO muteins.

B. Binding of EPO and EPO muteins to EPO Receptor

Measurement of binding affinity of EPO wild type and muteins to the receptor was basically similar to that of binding affinity of TPO and TPO muteins to TPO Receptor.

The binding affinity of EPO-[F148V] and EPO-[F142V] to the EPO receptor was increased compared to that of wild type EPO(Figure 4b). And the former mutein had the strongest binding affinity among all EPO muteins.

C. Binding of G-CSF and G-CSF muteins to G-CSF Receptor

Measurement of binding affinity of G-CSF wild type and muteins to the receptor was basically similar to that of binding affinity of TPO and TPO muteins to TPO Receptor.

Results(Figure 4c) showed binding affinity of G-CSF-[F140V], G-CSF-[F144V], and G-CSF-[F160V] to the G-CSF receptor was increased compared to that of wild type G-CSF. And the first mutein(G-CSF-[F140V]) had the strongest binding affinity among all G-CSF muteins.

D. Binding of GH and GH muteins to GH receptor

Measurement of binding affinity of GH wild type and muteins to the receptor was basically similar to that of binding affinity of TPO and TPO muteins to TPO Receptor.

Results(Figure 4d) showed that GH-[F139V] had the strongest binding affinity to the GH receptor.

EXAMPLE 6. Measurement of bindings of cytokines and their muteins to each of their receptors by using SPR

A. Binding of TPO and TPO muteins to TPO Receptor

To measure the binding affinity of TPO-[F141V] and TPO-[F131V] to TPO receptor, SPR was performed on a BIAcore 3000 instrument containing CM5 sensor chip. Anti-human IgG antibody was immobilized onto each flow cells 1 and 2 using amine-coupling chemistry. To inactivate any active group, surfaces were blocked with 1 M ethanolamine. TPO receptor-Fc fusion

protein was added to bind to the anti-human IgG antibody for 2 min at 30$\mu\ell$/min and then TPO and TPO muteins were reacted to bind to the TPO receptor.

At the same density of ligand, increased resonance unit (RU) means higher binding affinities. In Fig.5a, wild TPO, TPO-[F141V] and TPO-[F131V] were 10RU, 30RU and 20RU, respectively. This result showed that TPO-[F141V] had the strongest binding affinity. In addition, K_D values of wild type and mutein TPO were shown in Table 7.

<Table 7>

Changes of Binding-kinetic rate constant of wild type and mutein TPO

	$K_{on}(M^{-1}s^{-1})x10^5$	$K_{off}(S^{-1})x10^{-2}$	$K_D(\mu M)=K_{off}/K_{on}$	Chi^2	Relative Binding affinity
Wild type TPO	2.42	13.7	5.66	5.81	1
TPO-[F141]	12.8	0.51	0.04	6.03	141

B. EPO muteins

SPR was performed to measure binding affinities of EPO mutein-[F148V] and EPO-[F142V] with EPO receptor. Experimental procedure was similar to that for TPOs.

Fig. 5b was the SPR result of EPO wild type and muteins. In Fig. 5b, EPO-[F148V] showed 40RU, EPO-[F142V] 30RU. These results show that EPO-[F148V] had the strongest binding affinity. In addition, K_D values of EPO muteins were shown in Table 8.

<Table 8>

Changes of Binding-kinetic rate constant of wild type and mutein EPO

	$K_{on}(M^{-1}s^{-1})x10^5$	$K_{off}(S^{-1})x10^{-2}$	$K_D(\mu M)=K_{off}/K_{on}$	Chi^2	Relative Binding affinity
Wild type EPO	1.84	8.83	4.80	4.55	1
EPO-[F148]	14.0	0.64	0.05	2.26	105

EXAMPLE 7. Measurement of binding affinities of wild type- and muteins of cytokine by using FACS

A. Establishment of TF-1/*c-Mpl* cell line

TF-1/c-Mpl cell line was established by transfecting cDNA coding *c-Mpl* into TF-1 cell. Expression of c-Mpl was verified by using FACS analysis. The 1×10^6/ml of the TF-1/*c-Mpl* cells was washed with PBS buffer and purified *c-Mpl* mouse anti-human monoclonal antibody(BD PharMingen, USA) was incubated with the TF-1/*c-Mpl* cells. And then FITC-conjugated anti-mouse IgG(whole molecule; Sigma, USA) was added to verify expression of *c-Mpl* on surface of the TF-1/*c-Mpl* cells. As a result, graph of the TF-1/*c-Mpl* cell was shifted rightward from that of TF-1 cells. This result showed that *c-Mpl*, TPO receptor, was expressed on the TF-1/*c-Mpl* cell.

B. FACS analysis of TPO muteins

The 1×10^6/ml of TF-1/c-Mpl cell was suspended in PBS buffer and TPO wild type and –[F141V] was added to the suspension and incubated at 4℃ for 30-60 minutes, respectively. Biotinylated goat anti-human TPO polyclonal antibody (R&D, USA) was added to the cells above and incubated at 4℃ for 30-60 minutes. Streptavidin-FITC (Sigma, USA) was added to the cells above and incubated at 4℃ for 30-60 minutes. The cells were washed twice with PBS buffer to remove non-reacted Streptavidin-FITC. The cells were suspended in PBS buffer and flow cytometric analysis was performed at 488nm using EXCALIBUR(BD, U.S.A.).

In Fig. 6a, a binding curve of TPO-[F141V] was shifted rightward from that of wild type TPO. This result showed that TPO-[F141V] had much stronger receptor-binding affinity than the wild type TPO.

C. FACS analysis of EPO muteins

FACS procedure of EPO muteins was carried out similarly to that of TPO.

In Fig. 6b, a binding curve of EPO-[F148V] was shifted rightward from that of wild type TPO. This result showed that TPO-[F141V] is much stronger in receptor-binding affinity than the wild type EPO.

Example 8. Measurement of biological activities of TPO, EPO, G-CSF and GH muteins

A. Cell proliferation assay of TPO muteins

To investigate differences of cell proliferation and biological activities between TPO- wild type and muteins, TF-1/*c-Mpl* cell line produced above was used. TF-1/*c-Mpl* cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 1ng/㎖ GM-CSF at 37℃, 5% CO_2. 0.4, 1, 5, 10, 20, 40, 75ng/㎖ of each of TPO-wild type and muteins in RPMI-1640 were seeded in 96-well tissue-culture plates(FALCON, USA). 1×10^4 cell of the TF-1/*c-Mpl* cells in RPMI-1640 containing 10% fetal bovine serum was added to each wells of the 96-well plate. After 4 days cultivation at 37℃, 5% CO_2, 20 ㎕ of MTS solution[3-(4,5-dimethyl-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS] and the phenazine ethosulfate(PES;promega) was added and incubated for 4 hours. O.D. was measured with microplate reader(BIO-RAD Model 550) at 490 nm.

Figure 7a showed differences of TPO wild type and muteins in stimulating TF-1/*c-Mpl* cell proliferation. TPO was applied to the TF-1/*c-Mpl* from 0.4ng/㎖ to 75ng/㎖. Cell proliferation was increased up to 50ng/ml of TPO concentration. TF-1/*c-Mpl* cell proliferation potential of TPO-[F141V] was much stronger than that of wild type and was the first in biological activity among TPO muteins. Biological activity of TPO-[F131V] was the second strongest among TPO muteins. Activity of TPO-[F46V] was similar to that of wild type.

B. Cell proliferation assay of EPO muteins

Biological activity for EPO muteins was examined by cell proliferation assay using EPO-dependent TF-1 cell. Experimental procedure of cell proliferation assay of EPO muteins was similar to that of TPO muteins.

Figure 7b showed differences of EPO wild type and muteins in stimulating TF-1 cell proliferation. EPO was applied to the TF-1 Cell from 0.01IU/ml to 7IU/㎖. TF-1 cell proliferation potential of EPO-[F148V] was much stronger than that of the wild type and was the first in biological strength among EPO muteins. Biological activities of EPO-[F142V] and EPO-[F138V] were the second and the third strongest among EPO muteins, respectively.

<Table 9>

Biological activities of TPOs

	TPO	The maximum activity comparison(%)
Wild type		100
Muteins	TPO-[F46V]	107
	TPO-[F128V]	63
	TPO-[F131V]	119
	TPO-[F141V]	146

<Table 10>

Biological activities of EPOs

	EPO	The maximum activity comparison(%)
Wild type		100
Muteins	EPO-[F48V]	84
	EPO-[F138V]	57
	EPO-[F142V]	122
	EPO-[F148V]	137

C. G-CSF muteins

Biological activity for G-CSF muteins was examined by cell proliferation assay using G-CSF dependent HL-60 cell. Experimental procedure of cell proliferation assay of G-CSF muteins was similar to that of TPO muteins.

Figure 7c showed differences of G-CSF wild type and muteins in stimulating HL-60 cell proliferation. G-CSF was applied to the HL-60 Cell from 0.4ng/㎖ to 75ng/㎖. HL-60 cell proliferation potential of G-CSF-[F140V] was much stronger than that of the wild type and was the first in biological strength among G-CSF muteins.

D. GH muteins

Biological activity for GH muteins was examined by cell proliferation assay using GH dependent NB2 cell. Experimental procedure of cell proliferation assay of GH muteins was similar to that of GH muteins.

Figure 7d showed differences of GH wild type and muteins in stimulating NB2 cell proliferation. GH was applied to the NB2 Cell from 0.4ng/㎖ to 75ng/㎖. NB2 cell proliferation potential of GH-[F139V] was much stronger than that of the wild type and was the first in biological strength among GH muteins.

Example 9. Pharmacokinetic Profiles of EPO- and TPO- wild types and muteins

Difference of pharmacokinetic profiles of each EPO-and TPO- muteins between their wildtype was investigated. TPO or TPO muteins was injected intravenously into rabbits (NewZealand White, 3kg). And then blood samples were collected serially. EPO and TPO concentrations from each samples were detected by using quantitative ELISA assay as described above. Injection of EPOs into mice (12weeks, Balb/c, 30g) was performed by both intraperitonealy and intravenously. Blood samples in heparin-containing tubes were separated by centrifugation at 3,000rpm for 10 minutes. Supernatant containing plasma was used to detect blood concentrations of EPO and TPO by using ELISA.

After intravenous injection of 5 ㎍/kg of TPO wild type and –[F141V] into rabbit, plasma concentration profiles of TPO wild type and –[F141V] were shown in Figure 8a. Concentration of TPO-[F141V] was decreased more rapidly than that of wild type TPO. TPO-[F141V] was shifted from blood to peripheral target tissues more rapidly, due to its stronger binding affinity to receptor.

After intravenous injection of 1000 I.U/kg of wild type EPO and EPO-[F148V] into rabbit, plasma concentration profiles of wild type EPO and EPO –[F148V] in blood were shown in Figure 8b. Concentration of EPO-[F148V] was decreased more rapidly than that of EPO wild type.

After intraperitoneal injection of 20 I.U/g of wild type EPO and EPO–[F148V] into mice, plasma concentration profiles were shown in Figure 8c. The diffusion velocity of EPO wild type was

higher than that of EPO-[F148V] at early stage and maximum concentration in blood(Cmax) of wild type EPO was also higher than that of EPO-[F148V]. Cmax of EPO-[F148V] remained longer than wild type EPO. These results suggested that EPO-[F148V] was more hydrophobic and had higher binding affinity to receptor than the wild type EPO. And these results lead to the conclusion that EPO-[F148V] was diffused into blood more slowly and shifted from blood to peripheral target tissues more quickly than those of wild type EPO.

<Table 11>

Pharmacokinetic parameters of EPO wild type and EPO-[F148V] mutein

	Mouse		Rabbit	
	Wild type EPO	EPO-mutein [F148V]	Wild type EPO	EPO-mutein [F148V]
$T_{1/2}$(Half life)	1.9	1.4	3.8	2.4
AUC	100	78	100	80

Example 10. In vivo activities of EPO muteins

Difference of biological activities between EPO- wild type and muteins was verified in mice. Mice (12weeks Balb/c, 20g, Jungang Lab Animal Inc., Korea) were γ-irradiated at 700Rad. 250ng of purified EPO wild type and muteins in 50 $\mu\ell$ of PBS were injected intraperitoneally 3 times everyday. Blood samples were collected from their tail vein. And then hematologic parameters were tested according to ordinary CBC test. Wild type EPO was used as a positive control and CHO cell culture supernatant was used as a negative control. Blood was collected into tubes containing EDTA at 0, 1st, 2nd, 4th, 7th, 10th, 15th, 20th, 25th, and 30th days after the injection.

Figure 9 showed that CBC results in mice injected intraperitoneally with EPO- wild type and muteins to verify change in count of RBC and reticulocyte. Increase of RBC count (Figure 9a) was much more remarkable in EPO[F148V]-injected mice than mice injected with wild type EPO. And the RBC increase of in EPO[F48V]- and EPO[138V]- injected mice was weaker than that of mice

injected with wild type EPO. Increase of reticulocyte count (Figure 9b) and hematocrit was similar to the result of RBC count change in mice injected with EPO-[F148V].

Example 11. In vivo activities of TPO muteins

Difference of biological activities between TPO- wild type and muteins was studied in mice. Mice (12weeks Balb/c, 20g, Jungang Lab Animal Inc., Korea) were γ-irradiated at 700Rad. 250ng of purified TPO wild type and muteins in 50 ㎕ of PBS were injected intraperitoneally 3 times everyday. Blood samples were collected from their tail vein. And then hematologic parameters were tested according to ordinary CBC test. Wild type TPO was used as a positive control and CHO cell culture supernatant was used as a negative control. Blood was collected into tubes containing EDTA at 0, 1st, 4th, 7th, 10th, 14th, 18th 23rd, 28th, and 32nd days after injection.

Figure 10 showed the changes of platelet count(Figure 10a), leukocyte count(Figure 10b),and neutrophil count(Figure 10c) in mice injected intraperitoneally with TPO- wild type and muteins. Increase of platelet count was the most remarkable in mice injected with TPO-[F141V]. And mice injected with TPO-[F131V] was the second highest. Mice injected with TPO-[F46V] was similar to those injected with wild type TPO. And mice injected with TPO-[F128V] showed platelet count similar to that of negative controls injected with PBS (Figure 10a). Increase of leukocyte count (Figure 10b) and neutrophil count (Figure 10c) showed similar patterns as those seen in platelet change.

Industrial Applicability

As apparent from the above results of the present invention, valine substitution for phenylalanine residue, which is present in a domain participating in the binding of conventional wild-type biological response-modulating proteins to corresponding receptors, ligands or substrates, leads to an increase in binding affinity and biological activity, and reduces the production of autoantibodies to conventional protein variants, thereby making it possible to produce improved protein drugs.

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